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Germ-line mutations of the *APC* gene in 53 familial adenomatous polyposis patients

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ABSTRACT **We searched for germ-line mutations of the *APC* gene in 79 unrelated patients with familial adenomatous polyposis using a ribonuclease protection analysis coupled with polymerase chain reaction amplifications of genomic DNA. Mutations were found in 53 patients (67%); 28 of the mutations were small deletions and 2 were 1- to 2-base-pair insertions; 19 were point mutations resulting in stop codons and only 4 were missense point mutations. Thus, 92% of the mutations were predicted to result in truncations of the APC protein. More than two-thirds (68%) of the mutations were clustered in the 5′ half of the last exon, and nearly two-fifths of the total mutations occurred at one of five positions. This information has significant implications for understanding the role of *APC* mutation in inherited forms of colorectal neoplasia and for designing effective methods for genetic counseling and presymptomatic diagnosis.**

Familial adenomatous polyposis (FAP) is an autosomal-dominant inherited disease, affecting 1 in 5000 and 1 in 17,000 of the American and Japanese populations, respectively (1). FAP is characterized by the development of hundreds to thousands of adenomatous polyps in the colon and rectum, one or more of which can progress to cancer if left without surgical treatment. Neoplasia is not limited to the colon and rectum of patients with FAP, as some patients are affected with desmoid tumors, osteomas, fibromas, and various other neoplasms in addition to polyps.

Cytogenetic and linkage studies have localized the gene responsible for FAP to chromosome 5q21 (2–5), a region that is also deleted commonly in sporadic colorectal tumors (6–8). Hence, it was considered likely that germ-line or somatic mutations of chromosome 5q21 gene(s) would lead to adenoma formation in familial and sporadic forms (6).

Recently, several chromosome 5q21 genes were identified (9–13). One of them (the *MCC* gene) was shown to be somatically mutated in a subset of sporadic colorectal cancers. Another (the *APC* gene) was shown to undergo similar somatic mutations and also to be mutated in the germ-line of patients with FAP. *APC* and *MCC* were predicted to encode coiled-coil proteins that might interact *in vivo* with themselves or with other proteins.

In our initial study, we examined three exons of *APC* in 103 kindreds with FAP and found germ-line mutations in five of them (11). Groden *et al.* (12) examined a large portion of the *APC* gene product but found only four mutations in 61 separate kindreds. These studies thus left open the question of whether other genes might be involved in a substantial number of kindreds.

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To answer this question, and to further investigate the nature of inherited *APC* gene mutations, we have now examined the entire coding region of *APC* in 79 unrelated kindreds with FAP. We were able to identify presumptive mutations in 53 of these kindreds, suggesting that *APC* is responsible for the great majority of FAP cases. Remarkably, >90% of the mutations resulted in truncations of the predicted protein product. These results have significant theoretical and practical implications for understanding etiology and diagnosing disease in susceptible individuals.

MATERIAL AND METHODS

Genomic DNA of FAP Patients. FAP patients were identified on the basis of clinical manifestation. Genomic DNAs of 79 unrelated patients, including 55 American and 24 Japanese kindreds, were prepared from leukocytes as described (14).

PCR. The coding region of the *APC* gene was divided into 31 segments (see *Results*), and each segment was separately amplified using PCR (15). The primer pairs used in this study are listed in Table 1. PCR was performed with 38 cycles for 0.5 min at 95°C, 2 min at 51°C, and 2 min at 70°C as described by Baker *et al.* (16).

RNase Protection Analysis. RNase protection assay was performed by the method of Winter *et al.* (17) as modified by Kinzler *et al.* (9). Briefly, PCR products were hybridized to ^{32}P-labeled RNA transcripts corresponding to normal APC sequences, cloned, and labeled as described by Nishisho *et al.* (11). The hybrids were digested with RNase A, which cleaves at mismatches within DNA·RNA hybrids. The size of the digestion products was analyzed by polyacrylamide gel electrophoresis. Two separate RNase protection analyses were done by using sense and antisense strands.

Cloning and Sequence Analysis. PCR products showing abnormalities in RNase protection patterns were cloned into a plasmid vector (pBluescript SK Stratagene) as described (11). DNAs from a pool of at least 50 subclones were used as a template for each DNA sequencing reaction. DNA sequencing was carried out according to the method described by Nigro *et al.* (18). Sequencing of both strands of genomic DNA was performed to confirm a mutation.

RESULTS

We examined the entire coding region of the *APC* gene in the germ line of 79 unrelated FAP patients by an RNase protection assay. The coding region of the *APC* gene is contained within 15 exons (nos. 1–15) (9, 11), preceded by at least one 5′ noncoding exon (unpublished data). The coding region was

Abbreviation: FAP, familial adenomatous polyposis.

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Table 1. Sequences of primers used for PCR of RNase protection analyses

	Upstream primer	Exon	Codons		Downstream primer
G31	ATGGAATTCTTCTTAAACTGCTTAAGAG*	1	1–45	G18	TTTACAAGAGGGAATACTGAAT
G21	CCTGAATTCAAGAAATACAGAATCACGTC*	2	46–74	G22	ATGAAGCTTGTACTTGGATCTACACACC‡
G29	ATGGAATTCCATTAAGAATATTTTAGACTGCT*	3	74–141	G28	TTAAAGCTTAACAATAAACTGGAGTACACA‡
G27	ATGGAATTCCAACTGATGTAAGTATTGCTC*	4	141–177	G30	ATGAAGCTTTAATGGATTACCTAGGTACT‡
G17	CAGGAATTCTTTATTGGTTCTTATATGCT*	5	178–215	G26	CTGAAGCTTCCTAATAGCTCTTCGCTG‡
G23	CAAGGATCCTGAGCTTTTAAGTGGTAG†	6	216–243	G20	CTGAAGCTTTTCTCAGAATAACTACCTA‡
G19	ACTGAATTCCTTGGGCTAAGAAAGCCT*	7	244–278	G24	ATGAAGCTTCTTAGAACCATCTTGCTTC‡
G5	CATGATGTTATCTGTATTTACC	8	279–311	G4	CTTAGCAAAGTAGTCATGGC
G1	GGATATTAAAGTCGTAATTTTGT	9	312–438	G2	CATGCACTACGATGTACACT
G13	CATCATTGCTCTTCAAATAACA	10	438–470	G14	CACCAGTAATTGTCTATGTCA
G9	TAGATGATTGTCTTTTTCCTCT	11	470–516	G10	TCATACCTGAGCTATCTTAAG
G7	GCTTGGCTTCAAGTTGTCTT	12	517–542	G8	CAGAGTGAGACCCTGCCT
G11	GCAACTAGTATGATTTTATGTATAAA	13	543–581	G6	ACATGAAATTCATATTATAGTACT
G15	CAACTCTAATTAGATGACCCA	14	582–653	G16	GAGAGTATGAATTCTGTACTT
G35	CAATCATATTATGCCTTTTGTC	15-1	653–751	C22	GATGGCAAGCTTGAGCCAG
E9-1	CGAAGTACAAGGATGCCAAT	15-2	735–884	E9-2	CAGTGGTGGAGATCTGCAA
E9-3	AACTACCATCCAGCAACAGA	15-3	862–1022	E9-4	TCTAGTTCTCCATCATTATCAT
C23	TCAATACCCAGCCGACCT	15-4	998–1141	E9-6	GGCTTATCATCTTCATAGTCA
E9-5	GTAAGCCAGTCTTTGTGTC	15-5	1125–1284	E9-8	CAGCTGATGACAAAGATGAT
E9-7	AGACTTATTGTGTAGAAGATAC	15-6	1260–1410	E9-10	ATGGTTCACTCTGAACGGA
E9-9	TCTGTCAGTTCACTTGATAG	15-7	1389–1547	C36	CATTTGATTCTTTAGGCTGC
E9-11	ACAGAAAGATGTGGAATTAAG	15-8	1516–1673	E9-12	TTCTCCAGCAGCTAACTCAT
E9-13	GCTACATCTCTAAGTGATCT	15-9	1654–1826	E9-14	CTTATCATTGAAGTCCTTGG
E9-15	CTCAGACAACAAAGATTCAAA	15-10	1805–1965	C52	GAGAAAAGCAAACTGGAGTA
E9-17	AGTCATCCAAAGACATACCA	15-11	1935–2097	E9-22	CTGAATCAGGGGATAGACC
C35	GATATACAGAGACCAGATTCA	15-12	2082–2246	C38	ACAGGACTTGTACTTGAGGA
C37	CGAGGCAGGACAATGATTC	15-13	2226–2396	C40	GACTCACTTCTTGGAATACTA
C39	CAGATGAGCCAACAGAACC	15-14	2372–2472	C42	GCTGGTCTAGATGATGGAG
E9-19	TGGAGGAATCTGCTTCATTT	15-15	2456–2610	E9-16	TCCTTTTGCGGATACTTGG
E9-21	TGAACTCTATTTCAGGAACC	15-16	2592–2760	E9-18	GTACGTTCCACTATAGAACT
E9-23	TGTCCCTGTATCAGAGACT	15-17	2745–2843	E9-20	TGTCTATATAGCAGTTGTAATT

The amplified regions are indicated by exon and codons contained within each fragment. Exon 15 is divided into 17 overlapping segments, each ≈400 bp in length. Primers are described from the 5′ → 3′ direction and some of them include *Eco*RI (*), *Bam*HI (†), or *Hin*dIII (‡) sites within the primers to facilitate cloning.

divided into 31 segments. The first 14 segments corresponded to 14 individual exons. The coding region in the last exon (no. 15) is very large [6571 base pairs (bp)] and was divided into 17 overlapping segments, each ≈400 bp in length. These 31 segments were individually amplified from each of the 79 patients and subjected to RNase protection analyses as described in *Material and Methods*.

Fig. 1 presents examples of RNase protection analyses in which variations were observed. PCR products in which variations were detected were cloned into a plasmid vector and sequenced (examples in Fig. 2). Patient 100 had a C → A transversion at the second nucleotide of codon 932, resulting in a change from serine (TCA) to a stop codon (TAA) (Fig. 2*A*, lane 2). Patient 16 showed a 2-bp deletion (AG) of codon 1465 (AGT) (Fig. 2*B*, lane 2). A T insertion at the second nucleotide of codon 1211 (ATTG) was found in patient 39 (Fig. 2*C*, lane 2, beginning at arrow).

The results from the PCR-RNase protection analyses are summarized in Table 2. Presumptive mutations altering the sequence of the predicted protein product were observed in 53 of the 79 patients studied. No patient had more than one of these mutations, and none of these mutations was observed in the germ-line DNA of at least 100 individuals without FAP studied by RNase protection assay or direct sequencing of PCR products.

Nature of Mutations. As summarized in Table 3, 23 of the 53 alterations were point mutations. Nineteen of them generated stop codons. Two of the four amino acid changes were nonconservative (resulting in the substitution of cysteine for serine or arginine), and two were functionally conservative (serine to threonine or leucine to phenylalanine, respectively). Twenty-one of the 23 mutations resulted in a change from C to some other nucleotides in which C → T was the most common (Table 4). The point mutations were scattered throughout the gene and no "hot spot" was detected; only the point mutations at codon 302 and 625 were observed in more than one kindred (Table 2).

Thirty of the 53 mutations were associated with frameshifts due to deletions (28 cases) or insertions (2 cases) (Table 3). Deletions of 1–5 bp were observed, with 5 bp being most common (18 of the 28 deletion mutations involved 5 bp). Interestingly, a 5-bp deletion at codon 1309 was observed in 10 separate kindreds; this was by far the most common genetic alteration observed among the families. All of the deletion and insertion mutations altered the reading frame and created stop codons immediately downstream.

Distribution of Mutations. The distribution of mutations in the *APC* gene in the 53 FAP patients is shown in Fig. 3. Point mutations were found in exons 5, 6, 8, 9, and 12–15. However, all but one of the 30 cases of deletion or insertion were found in exon 15 (one case was at the exon–intron junction of exon 4). Thirty-six (68%) of the total mutations (including point mutations and frameshifts) were clustered within the 5′ half of exon 15 (codons 713–1597), representing less than one-third of the coding region. Five specific mutations were found in more than one unrelated kindred (Table 2 and Fig. 3).

DISCUSSION

Most of the mutations described in this study are predicted to have profound effects on the predicted gene products. Forty-nine (92%) of the mutations led to incomplete products of the *APC* gene due to translational termination; 19 of the 23 point mutations were nonsense mutations and all 30 frameshift mutations created new stop codons immediately downstream. Missense mutations were observed in only four patients. In two of these four cases, the change to cysteine from arginine or serine is expected to have significant effects on the structure of the predicted protein. The other two changes were relatively conservative, and we cannot be sure

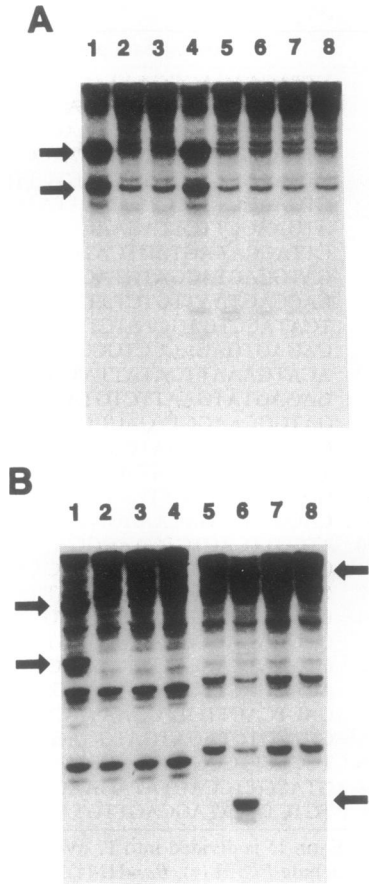

FIG. 1. RNase protection analyses. (*A*) Lanes 1 and 4 show the same variant RNase protection patterns (arrows) in patients 5 and 10, respectively, using the exon 15-4 probe (Table 1). (*B*) Lanes 1 and 6 represent different abnormal patterns (arrows) in patients 15 and 91, respectively, observed with the exon 15-5 probe (Table 1).

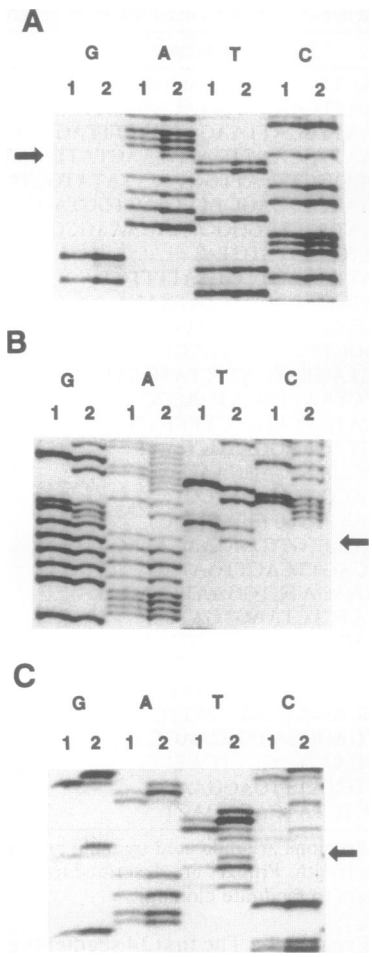

FIG. 2. Sequence analyses of PCR products from normal and affected individuals. Patient 100 (*A*, lane 2) shows a C → A change (arrow) at the second nucleotide of codon 932 resulted in a change from serine (TCA) to a stop codon (TAA). Patient 16 (*B*, lane 2) shows a 2-bp (AG) deletion of codon 1465 (AGT) and patient 39 (*C*, lane 2) shows a T insertion at the second nucleotide of codon 1211 (ATTG) beginning at the arrow. Extra bands can be seen after deletion or insertion in addition to normal bands. The reaction samples of different individuals are grouped so that mutations can be easily recognized.

that these substitutions represent true mutations rather than rare variations with no functional effect. However, this serine to threonine (patient 3) or leucine to phenylalanine (patient 89) change was the only change detected by RNase protection analyses of the entire coding region, except one amino acid polymorphism of patient 3 (described below). As the biochemical and physiologic properties of the APC protein are unknown, these missense mutations might prove to be valuable for assessing function and to provide clues for localizing the critical effector domains of the very large protein encoded by the *APC* gene.

Ninety-one percent of the 23 point mutations resulted in a substitution for C, most commonly with a T (Table 4). These occurred at seven CA sites, five CG sites, and one CT site. Deamination of 5-methylcytosine in the CpG dinucleotide has been implicated as a mechanism for point mutation from C to T (19). But only five of our cases involved CpG sites (Table 2). The mechanism for CpA to TpA and CpT to TpT change is not known. However, as the most common DNA polymerase error is thought to be a G mispairing with T (20) with a lack of repair at this mismatch (21), the CpA to TpA and CpT to TpT mutations we observed might have been generated in this manner.

Twenty-eight of the 30 cases with frameshifts were associated with small deletions (Table 2). It is well known that deletions occur at repeated bases, perhaps because of misalignment; for example, we observed a C deletion from CCC (at codon 1427), an A deletion from several As (codon 142), and an AA deletion from AAA (codon 1250). A model for generating misalignment within a stretch of common bases has been proposed (22, 23). Some deletions were observed at positions containing several copies of a direct repeat; for example, an AG deletion was detected at the sequence of AAAGAGAGAGAGTG (codon 1465), an AAAGA deletion at ATAAAAGAAAAGATT (codon 1309), an ACAAA deletion from ATAAAACAAAGT (codon 1061), and a TGAAA deletion from TCAAATGAAAAC (codon 1546). All of these deletions might have occurred during DNA replication as a result of slippage of the template strand and subsequent misalignment (24). After synthesis of the first copy of the direct repeat, the template strand could slip and misalign with the second copy of the repeat, resulting in deletion of the intervening sequences.

All but one of the frameshift mutations due to insertions or deletions were detected within exon 15, but point mutations were scattered throughout exons 5–15. The most frequent mutation was observed at codon 1309. As this mutation was observed in Caucasian, Black, and Japanese populations, a founder effect is excluded.

In the course of RNase protection analyses, we found several polymorphisms within the coding sequences resulted with and without amino acid changes. Four major polymorphisms are TAC/TAT (at codon 486), GCA/GCG (at codon

Table 2. Germ-line mutations in the *APC* gene of 53 unrelated FAP patients

Patient(s)	Codon	Nucleotide change	Amino acid change
13	142	aatag/GTC → atag/GTC	a deletion
102	213	CGA → TGA	Arg → stop
11	215	CAG → TAG	Gln → stop
33	232	CGA → TGA	Arg → stop
93*	280	TCA → TGA	Ser → stop
24*, 34*	302	CGA → TGA	Arg → stop
21*	414	CGC → TGC	Arg → Cys
7	541	CAG → TAG	Gln → stop
90	577	TTA → TAA	Leu → stop
86	622	TAC → TAA	Tyr → stop
8, 38, 66	625	CAG → TAG	Gln → stop
60*	713	TCA → TGA	Ser → stop
3	784	TCT → ACT	Ser → Thr
49	794	AGTC → ATC	G deletion
84	806	CATGA → CGA	AT deletion
80	827	AAT → AAATT	AT insertion
124	857	GGAATTGG → GGG	GAATT deletion
100	932	TCA → TAA	Ser → stop
62	1055	ATAATAGA → AGA	TAATA deletion
5, 10, 70, 103	1061	AAACAAAG → AAG	ACAAA deletion
104	1102	TAC → TAG	Tyr → stop
91	1156	GAAGAGA → GGA	AAGA deletion
43	1175	CAG → TAG	Gln → stop
15	1191	CAGA → CAA	G deletion
39	1211	ATG → ATTG	T insertion
1	1230	CAG → TAG	Gln → stop
47	1249	TGC → TGA	Cys → stop
51	1250	AAAGT → AGT	AA deletion
6, 17, 20, 22, 25, 29, 46, 57, 59, 61	1309	GAAAAGAT → GAT	AAAGA deletion
120	1427	CCTG → CTG	C deletion
16	1465	GAGTG → GTG	AG deletion
28, 78	1546	AATGAAAA → AAA	TGAAA deletion
79	1567	TCA → TGA	Ser → stop
85	1597	ACTG → ACG	T deletion
19	2621	TCT → TGT	Ser → Cys
18	2644	ATTTATC → ATC	TTAT deletion
89	2839	CTT → TTT	Leu → Phe

Lowercase and uppercase letters indicate intron and exon, respectively.
*Reported previously (11).

545), ACG/ACA (at codon 1493), and GGA/GGG (at codon 1678). All of these polymorphisms have not changed coding amino acids—tyrosine, alanine, threonine, and glycine, respectively. The first polymorphism of the tyrosine coding sequences creates restriction fragment length polymorphism with *Rsa* I. This polymorphism and the last glycine coding polymorphism are the same as reported previously (12). The allelic frequencies of these polymorphisms ranged from 0.36 to 0.64 (data not shown). Five other polymorphisms, with allelic frequencies of <0.01, were also recognized. Two resulted in amino acid substitution—ATA (isoleucine) to GTA (valine) at codon 1304 of patient 6 and GGT (glycine) to AGT (serine) at codon 2502 of patients 3 and 70. Because nos. 6 and 70 contained other mutations that had profound effects on the predicted gene products (Table 2), we think these amino acid changes probably do not alter the function of the predicted proteins. The other three rare polymorphisms were TTG/TTA at codon 548 of patient 90, ATA/ATT at codon 1055 of patient 22, and CTA/TTA at codon 2401 of patient 1; none of these resulted in amino acid changes.

We detected mutations in 67% of FAP patients using an RNase protection assay. We did not find mutations in all patients for the following reasons. (*i*) Some mismatches are protected from RNase digestion. In fact, the reported sensitivity of detecting mutations with RNase is only 35–50% (11, 25, 26). We attribute our higher success rate to the fact that so many of the mutations in the *APC* gene were small deletions or insertions, which are usually quite susceptible to digestion with RNase A. (*ii*) The promoter region of the *APC*

Table 3. Frequency of germ-line mutations in the *APC* gene

Mutation	2
Point	
Nonsense	19
Missense	4
Frameshift	
Deletion (1–5 bp)	28
Insertion (1–2 bp)	2
Total	53

Table 4. Summary of point mutations in the *APC* gene

From/to*	C	T	G	A	Total
C	—	13	5	3	21
T	0	—	0	2	2
G	0	0	—	0	0
A	0	0	0	—	0
Total	0	13	5	5	23

*Listed in coding strand.

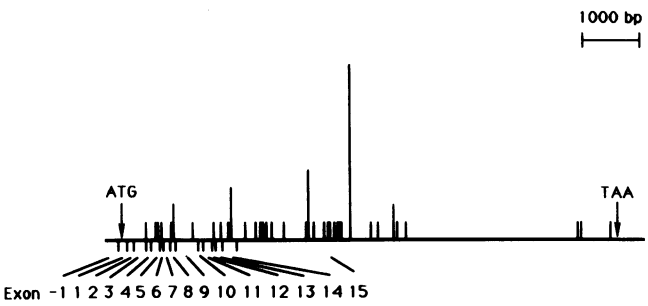

FIG. 3. Distribution of germ-line mutations in the *APC* gene. The length of the bar indicates the number of mutations at the indicated position. Positions of translational initiation (ATG) and termination (TAA) codons are marked with arrows.

gene has not yet been examined. (*iii*) Nonexamined sequences within introns may have a significant influence on gene expression. (*iv*) There may be a second FAP gene. We think, however, that the latter possibility is now unlikely.

The results of the studies described above provide significant insights into the nature of the mutations leading to FAP. They suggest that the carboxyl terminus is required for function, because deletions that remove this end of the protein (including a deletion that removes only the last 200 amino acids of the 2843-residue protein) result in disease. Further studies will be required to determine whether specific mutations are associated with specific phenotypes (such as early age at onset or a high prevalence of extracolonic neoplasms). However, these studies already provide a basis for presymptomatic diagnosis. In 53 kindreds reported here, such diagnoses can now be made with virtually 100% accuracy simply by testing for the relevant mutation. In kindreds not yet studied, it would seem advisable to begin screening for mutations at the five positions accounting for nearly 40% of the total alterations detected (Table 2). If these were negative, the next logical step would be to examine the 5′ half of exon 15, which contained more than two-thirds of the mutations. Only if this failed would an analysis of the remainder of the gene be warranted. Finally, these studies suggest an alternative method of examination for *APC* mutations. Ninety-two percent of the total mutations detected are predicted to result in truncated protein products. Thus, detection of these shortened proteins using antibodies against the APC protein is likely to be a valuable screening method in a high fraction of kindreds.

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